ALFA PATENT

PCT/TR03/00019

COMMENTS ON THE 1ST WRITTEN OPINION

The Written Opinion on PCT/TR03/00019 dated 25th January 2005 states that claims 1-5 and 10 lack novelty and that the subject matter of construct claims 6-9 and the subject matter of method claims 11-16 lack inventive step. The industrial applicability of the claims has not been questioned. The above opinion is based on the following documents:

D1: WO 99/ 57997

D2: DE 100 13 204

D3: Cha Hyung Joon et al, Biotechnology and Bioengineering 2000, John Wiley & Sons Inc. New York, NY, USA Vol 67, no 5, 2000, pages 565-574

D4: Keefe Anthony D et al, Protein Expression and Purification, vol. 23, no. 3, December 2001, pages 440-446

AMENDMENT

In view of the Written Opinion the applicants have amended the claims in that

New Claim	Support
1	Old claims 1 and 6; specification page 4 lines 19-20
2-5	Old claims 2-5
6-8	Old claims 7-9
9	Old claims 10 and 11
10-14	Old claims 12 –16
15	Specification page 3 line 33-page 4 line 4 and old claims 10-16
16	Specification page 3 line 33-page 4 line 4 and old claims 1-16

NOVELTY

The new main construct claim 1 has been amended to include the subject matter of old claim 6 whose novelty has been acknowledged by the Examiner. Therefore the new main construct claim 1 as well as claims 2-8 dependent thereon are also novel.

The new main method claim 9 has been amended to include the subject matter of old claim 11 whose novelty has been acknowledged by the Examiner. Therefore the new main construct claim 9 as well as claims 10-14 dependent thereon are also novel.

The new claim 15 relates to a two component system obtained by the method claims. Therefore this claim as well as new claim 16 directly dependent thereto are also novel.

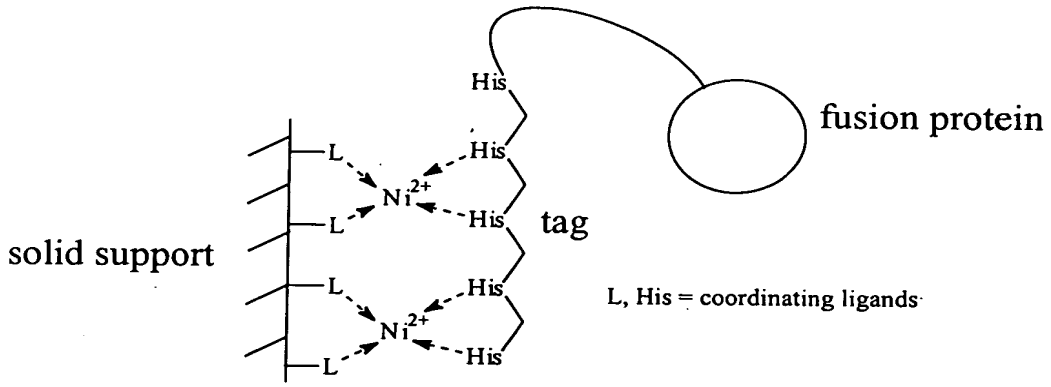
INVENTIVE STEP

THE CONSTRUCT CLAIMS

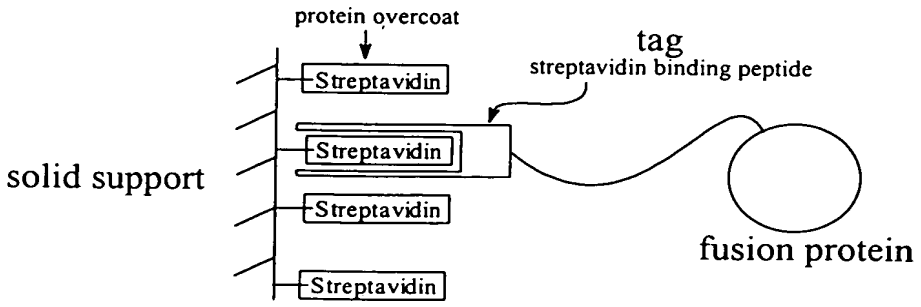
The Examiner states that: *"The application aims to provide alternative to conventional His-Tag-Ni-Cellulose purification techniques. However, claimed are vectors that comprise the same elements as already known in the prior art."*

The applicants do not agree with the Examiner in that their vectors do not comprise the same elements as already known in the prior art and in particular do not comprise metal ions and hence the binding mode between support and tag in the invention is different. In this discussion, three binding modes have been considered between support and tag:

1) **Non-protein surface bound to a His-tag through a bridging metal ion.**
   At least two coordinating ligands, one on the tag and one along the surface, and a compulsory bridging metal ion, typically Ni^{2+}, comprise this type of interaction. The interaction described in prior art documents D1-D3 (and marginally in D4) is based on this mode of binding (figure below). The interaction described in the present invention is not.

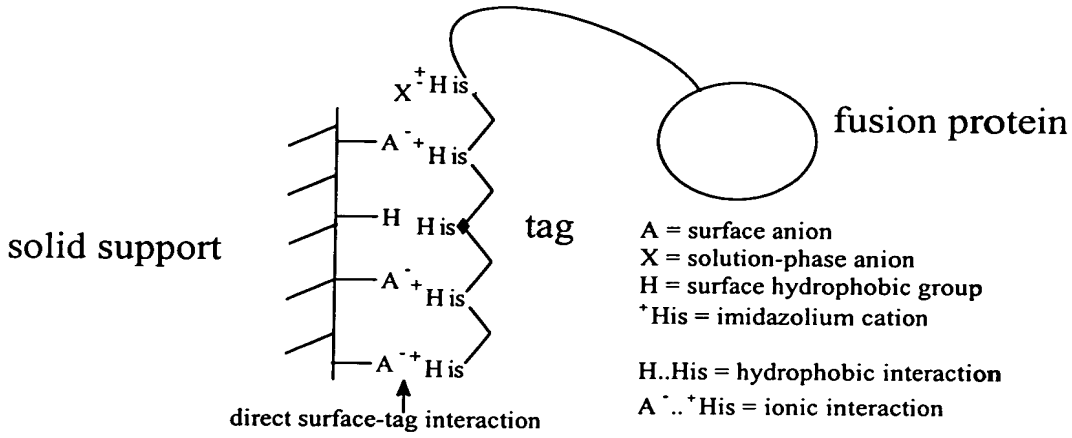


2) **Protein surface bound to a protein substrate tag.**
   This type of interaction is direct (tag-to-surface) and non-covalent but requires a highly specific protein linked along the support surface and an equally specific tag. The interaction emphasized in prior art document D4 is based on this mode of binding (figure below). The interaction described in the present invention is not.



3) Non-protein surface bound directly to a tag.

This type of interaction is always direct (tag-to-surface), generally non-covalent, optionally covalent, and its formation precludes any need of protein along the surface. A great variety of simple surface and tag functional groups can be selected to form this interaction. The present invention exploits this type of binding interaction. The modes of binding described in prior art documents D1-D4 is not based on this type of interaction (compare elements of the first and last figures). In addition, the present invention does not require any protein-layered support material whereas the method outlined in prior art D4 requires an extremely specialized protein (compare elements of the 2nd and last figures).



Further discussion:

Mode 1 versus mode 3. His-tag-Ni-support interactions are realized through the metal coordinating property of certain ligands (see prior art documents D1-D4). By way of this interaction, a poly-his tag is made to bind onto a surface through a bridging nickel ion, typically for purification purposes. The selection of available surface-tag partners depicted in prior arts D1-D4 is limited by virtue that the interaction is realized through a coordinated nickel ion and thus the surface and tag selected must bear coordinating (ligand-like) traits. In contrast, the invention does not use metal ions at all and binding of tag to surface does not arise indirectly through a bridging metal ion, nor is the interaction based on any related form of coordination chemistry. Instead, the two modes of binding exemplified in the invention are based on columbic interactions, and to some extent, base stacking. Many other modes of binding and simultaneous combinations thereof are potentially available by virtue of the invention's design flexibility so that the choice of interaction is not limited to columbic interactions and base stacking. Moreover, the uniqueness of the present invention's approach is in its presentation of a protein-friendly, non-destructive, yet direct protein immobilization strategy, which relies on genetically designed, modified and engineered constructs to achieve protein immobilisation in place of implementing potentially harsh *in vitro* chemistries, which are traditionally applied.

Mode 2 versus mode 3. The streptavidin-streptavidin binding peptide type of strategy has been emphasized in prior art document D4. Clearly, the physico-chemical constraints of this highly specialized interaction immediately limits the choice of surface and tag elements, as this protein-guest type of interaction is very much dependent on the physical shapes and not just the chemical compatibility of surface and tag. In comparison, the invention exploits a much more flexible binding strategy, whose possibilities are only restricted by the appropriate selection of favorably interacting surface groups and tags. The interactions formed are governed only by the chemical compatibility between potential bonding partners. The interactions are not shape-sensitive. In fact, the flexible tag can potentially conform to the shape of the surface, yielding a composite bond between multiple hydrophobic-hydrophobic groups, cationic-anionic groups, etc.

The mode of binding described in prior art D4 in comparison to the invention also differs greatly in strength. The streptavidin-streptavidin binding peptide interaction describes one of the strongest non-covalent interactions. It realizes high binding energies by exploiting a physico-chemical interaction that is based on the biologically evolved and optimized principle of host-guest shape complementarity, and its strength in reference D4 is cited as being on the order of $10^9 M^{-1}$ per protein-peptide interaction.

Cationic-anionic and hydrophobic-hydrophobic interactions are comparatively much weaker. Clearly, the strength of the surface-tag interaction in the present invention does not originate from shape-complementarity as in the case of the aforementioned prior art, but rather from the large number of simultaneously interacting groups positioned along surface and tag. Since the nature of the streptavidin-streptavidin binding peptide interaction is not in any way representative of typical surface-tag interactions, the binding mode specified in prior art document D4 should not be allowed to form a basis for which to argue against the novelty of binding modes depicted in the present invention.

To reiterate, the idea of achieving permanent immobilisation (not temporarily for purification purposes) of ANY peptide or protein of interest by exploiting the biosynthetic potential of its harvested or chemically synthesized gene sequence describes a very versatile, powerful and unique protein/peptide-friendly approach to immobilize enzymes, signal peptides, antibiotics, growth factors and relevant peptides onto appropriately tailored (not necessarily metal ion bearing) surfaces. The invention differs from other immobilization strategies in that **any** protein may be biologically synthesized along with ANY polyamino acid tag to attach the protein onto **any** number of appropriate synthetic surfaces, **without** the need of harsh chemical treatments or a **compulsory** his-tag.

The Examiner states that: *"Document D1 provides vector constructs comprising GFP, a MCS and an affinity peptide. The affinity peptide aims for the purification of the protein that is to be expressed by the vector construct. Said affinity peptide is specifically mentioned to be a histidine rich polypeptide sequence."*

The similarities professed by the Examiner are misconstrued. The vector construct of prior art document D1 and the vector construct of the present invention are not comparable beyond the use of GFP, MCS and affinity peptide terminology. The actual GFP, MCS and AP arrangement is notably different from the prior art and if the present invention's frame adaptor component is also considered, the novelty of the vector construct is self-apparent.

Further: The present invention's vector has been re-engineered to the extent that its design is not only different from any prior art, but also not an obvious extrapolation to an expert in the field. In particular, an expert would typically remove the MAD coding sequence and insert a small DNA sequence to put the GFP gene into frame, resulting in translation of the wrong peptide strand. In order to ensure the correct coding of a gene, its start position should also be put in the same reading frame with the overall stop of the fusion. In the present invention, this subtlety has been addressed. Namely, correct transcription of the gene has been achieved via insertion of an appropriate frame adapter component. In addition to the key function above, the gene component coding for the frame adaptor peptide is specifically designed to display a flexible linker that functions as a separator to mimic solution phase kinetics rather than immobilized kinetics, a feature, which may prove advantageous in certain cases.

The only plausible common ground between prior art document D1 and the present invention is related to the potential traceability of both vectors back to a common starting material. Any objection related to this possibility is unfounded, however, as the hypothetical parent vector, which would bare an outdated design, would not serve as a valid reference point. Even if this hypothetical parent vector did exist, or in other words, if the parent vector originated from the same plasmid organism, it would have been re-engineered along different paths and nearly beyond recognition in the course of taking on the specific designs described in prior art document D1 and in the invention.

The Examiner states that *"Also document D2-D4 provide for vector constructs with a visual marker protein like GFP, a multiple cloning site, and protein tags like a His-tag or a streptavidin binding protein. All disclosed vector constructs have the property that they can produce fusion-protein that can be further immobilized, visualized, and quantified. Consequently, D1-D4 all destroy the novelty of the product claims 1-5. Dependent claims 6-9 do not contain any features which, in combination with the features of any claim to which they refer, meet the requirements of the PCT in respect of inventive step."*

It appears that crucial elements of the original patent application were noted in the main body but not emphasized in the claims section, leading to a "domino effect" and dissenting review. With respect, the revised claims should ameliorate all concerns of the Examiner.

Further: The purpose of the following discussion is to outline distinctions between the present invention and prior art, and to touch upon some potentially contradictory aspects of the Expert's argument. Firstly, the common use of his-tags has left an impression. All cited prior art documents disclose the use of histidine tags. The high visibility of histidine tags is not surprising, as prior art D1 exclusively targets the chromatographic purification of proteins via metal-ion affinity, and specifically highlights the bridging role of a coordinating Ni ion. In fact, prior art documents D1-D3 are exclusively limited to the use of histidine tags. Document D4 in particular compares and contrasts aspects of the streptavidin-streptavidin binding peptide and the traditional polyhis tag-Ni^{2+}-surface interaction. At first glance, an overlap between previously disclosed polyhis tag technologies and the present invention would seem plausible. However, the present invention features one crucial distinction, which clearly separates the poly-his component from those expressed in prior art documents D1-D4. Namely, the present invention is not restricted to the use of a polyhis tag and even if a polyhis tag were employed, it would not use a Ni ion.

Secondly, the tag can be any polyamino acid, which interacts with the surface to form an ionic, polar, covalent or hydrophobic bond. This development is clearly inventive, as its conception was not obvious. For example, no mention of such a concept was expressed in any of the prior art documents D1-D4. Thus it would appear that the research underlying these developments was carried out in the belief that a histidine-metal ion or a streptavidin-streptavidin binding peptide partnership was necessary. If the principle of the present invention were clearly obvious to an expert, as the Examiner professes, then it would have been a short digression for researchers, who developed the technology of the prior art, and even some competitors, to validate the flexibility and merit of a metal-free, streptavidin-free interaction and to incorporate the multiple-mode, collective binding principle of the present invention into their technology.

Thirdly, affinity chromatography predates the specific example of metal-ion affinity chromatography. If the argument presented by the Examiner applied, it would follow to reason that the novelty of a metal-ion affinity claim should also be destroyed. Despite this assertion, metal affinity techniques have been patented in their own right. The same line of reasoning can be formed from yet another viewpoint. In particular, much literature has been published in which proteins are bound to surfaces via specific binding-site-to-surface interactions. Enzymes have been bound via surface immobilized substrates. Hormones have been bound to surface-immobilized receptors. It would appear that if an

expert, relying on the prior art, could have anticipated and reproduced the present invention, then by analogy, other individuals should have reproduced a method to purify fusion proteins via metal-ion affinity. Such a development in turn would have negated many potential patents, and yet, those cited as prior art documents D1 and D2 remain.

Fourthly, the present invention can function using any non-biological surface. The need to immobilize streptavidin or any related protein onto a synthetic support defines a crucial distinction between prior art document D4 and the present invention.

Fifthly, the applicants envisaged the use of variable amino acid sequences in the tag region to bind appropriately engineered synthetic surfaces via a variety of favorable interactions. Each interaction, irrespective of its nature, is chosen to yield a direct bond between surface and tag. Consequently, the method of the present invention does not use bridging metal ions. The approach of the present invention cannot be considered equal or similar to the prior art or any established column-immobilized Ni^{2+} chromatographic application, as the latter methodologies function exclusively through the coordination of metal ions, surface groups and his-tags. Examples listed in the present invention point out the merit of binding tag to surface via columbic interactions and base stacking. These interactions are unrelated to the chemistry of coordination complexes.

Sixthly, the His-tag - metal interaction and the streptavidin binding protein-streptavidin interaction of the prior art documents describe two highly specific interactions. Neither one has been addressed in the current invention. The interaction in the current invention is only limited by the choice of polyamino acid tag, which may be very diverse, and any suitably interacting surface onto which binding may be realized.

Finally, the frame adaptor is an essential part of the construct, which bridges the tag and GFP encoding regions of the construct. This is necessary at the DNA design level to re-establish correct reading frame and facilitate insertion of the target gene and is important at the protein level to facilitate surface-protein separation and the possibility of retaining native-like structure and bioactivity. In this respect, its role is inventive in the context of technologies established for the expression of fusion proteins.

To summarize, the his-tag presented by the inventors is held to the surface via metal-free bonding. Specific emphasis of the his-tag should not mislead, however, as the invention can incorporate any combination of positively charged, negatively charged, polar, and hydrophobic polyamino acid tags and in this sense the invention is completely unrelated to the prior art, which by virtue or coordination bonding, necessitates the use of his-tags or by virtue of extremely specialized protein-substrate interactions necessitates the use of streptavidin and streptavidin binding peptide, respectively. Applicants therefore believe that the amended construct claims are inventive.

THE METHOD CLAIMS

The Examiner states that: "*Document D4 discloses a construct as described supra, said construct is used in method of purification of a protein. The document explicitly refers to the streptavidin binding protein that can be used for detection of the recombinant .....Steps d,g and h are optional and have therefore no limiting effect to the claimed method. Therefore the method of claim 10 is not new (Art. 33(2)PCT)*

Dependent claims 11-16 do not contain any features which, in combination with the features of any claim to which they refer, meet the requirements of the PCT in respect of inventive step"

Applicants believe that this statement is not correct.

The main method claim has been amended by adding a characterising part specifying the support material. Moreover the present invention precludes any need of metal ion. In fact the present invention does not use any metal ion or streptavidin-streptavidin binding peptide.

Further discussion: The present invention describes a flexible and inexpensive alternative to immobilize and purify fusion proteins. The streptavidin-streptavidin binding peptide interaction is one of the strongest non-covalent interactions known. In fact, the interaction is so strong that it may be regarded as introducing potential limitations. For example, an expensive specific ligand such as biotin is required to displace streptavidin-binding peptide from a column. Consequently, the use of streptavidin binding peptide in a purification kit can have limitations in comparison to the current invention, whose flexible design could facilitate the elution of fusion proteins by the addition of standard chemicals. If immobilization with guaranteed retention is required, the present invention can be adopted to allow the formation of covalent bonds between any reactive surface and nucleophilic tag.

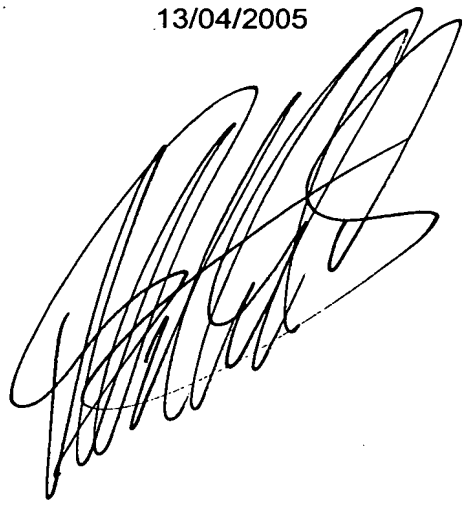
In readdressing the Examiner's comment, it is noteworthy that the streptavidin- streptavidin binding peptide interaction describes a very specialized form of binding, the most striking indication being the specific conditions necessary to dissociate a surface from tag. This highly specific interaction is based on the biologically optimized principle of host-guest shape complementarity. Cationic-anionic and hydrophobic-hydrophobic interactions are comparatively much weaker and less specialized. Clearly, the strength of the surface-tag interaction in the present invention cannot originate from shape-complementarity. Instead, the strength comes from a large number of simultaneously interacting groups positioned along surface and tag.

Since the nature of the streptavidin-streptavidin binding peptide interaction is not in any way representative of typical surface-tag interactions, the binding mode specified for streptavidin in prior art document D4 should not be allowed to form a basis for which to argue against the novelty of any binding modes depicted in the present invention.

Applicants therefore believe that the amended method claims are inventive.

In view of the amendments made in the claims and the comments given hereabove the Examiner is respectfully requested to reconsider the original opinion and to issue a positive examination report.

Yours truly
Selda ARKAN
13/04/2005



AMENDED CLAIMS

1) Circular recombinant plasmid DNA construct encoding a) a protein tag, b) a visual marker protein, and containing c) a multiple cloning site suitable for insertion of an additional gene, characterised in that it further contains d) a frame adapter of variable length between the visual marker and protein tag genes and in that the gene sequence encoding the protein tag, the visual marker protein and the frame adaptor are specifically designed and engineered at the DNA level for respectively i) immobilisation purposes, ii) visualisation and quantification purposes at the protein level, and iii) providing a large distance separating protein and surface to enable the immobilised enzymes to display native-like characteristics.

2) Construct according to claim 1, characterised in that the protein tag is chosen from the group containing lysine (lys), histidine (his), tyrosine (tyr), phenylalanine (phe), arginine (arg), glutamic acid (glu), aspartic acid (asp), glutamate, aspartate, asparagine (asn), glycine (gly), glutamine (gln), alanine (ala), valine (val), tryptophan (trp).

3) Construct according to claim 1 or 2, characterised in that the protein tag is a histidine-tag such as a polyhistidine variant, in particular (6X) histidine.

4) Construct according to any one of the preceding claims, characterised in that the visual marker protein is chosen from the group containing fluorescent or phosphorescent proteins, wherein the fluorescent protein is chosen from the group containing Green Fluorescent Protein (GFP), Red Fluorescent Protein (RFP), Yellow Fluorescent Protein (YFP) and Blue Fluorescent Protein (BFP) as well as their variants and/or mutants.

5) Construct according to any one of the preceding claims, characterised in that the multiple cloning site contains restriction enzyme recognition sites chosen from the group containing SacI, Sal I, Hid III, Eag I, Not I.

6) Construct according to any one of the preceding claims, characterised in that it expresses a fusion protein, wherein the tag is suitable to interact directly with appropriate surface pendant groups of a support matarial.

7) Construct according to claim 6, characterised in that the direct interaction with the support material is covalent or non-covalent.

8) Construct according to claim 7, characterised in that the direct interaction is non-covalent and yet freely accessible and leach-free like covalent one.

9) Method for preparing and immobilising a protein on a support material, comprising:
   a) Engineering at the DNA level, in series a protein tag suitable to interact directly with appropriate surface pendant groups of a support material, a fluorescent marker

protein for visualisation and quantification purposes at the protein level and a multiple cloning site suitable for insertion of a target protein to be immobilised,

b) Inserting the corresponding gene of the target protein to be immobilised into the multiple cloning site;

c) Initiating protein expression.

d) Optionally pre-treating the support material;

e) Incubating the protein and support material together, wherein the protein is immobilised to the support via specific tag-surface interactions;

f) Washing away the non-specific biomolecules;

g) Optionally quantifying the fluorescence of the visual marker protein;

h) Optionally desorbing the target protein.

characterised in that the support material is chosen from the group containing polymers, biopolymers, glass and composites containing silicone dioxides, metals and metal oxides, as well as any combination thereof on the microscopic, mesoscopic or macroscopic length scale.

10) Method according to claim 9, characterised in that the support material is chosen from the group containing polymers, silicon dioxides, aluminum oxides, titanium oxides, magnesium oxides, borates, metals and other metal oxides.

11) Method according to any one of claims 9 and 11, characterised in that the polymers are chosen from the group containing polyolefins such as polystyrene, polyacrylates, polymethyacrylates, polybutylene, polyvinylalcohol and related derivatives, polyvinylchlorides, polyisoprene, polypropylene, polyphenols, polyamides, polyesters polysulfones, polyethersulfones, polyethersulfides, polyimines, polyethyleneglycols, polypropyleneglycols, polyimides, polycarbonates, polyurethanes.

12) Method according to claim 11, characterised in that the polymer surface is chemically treated to bear various functional groups chosen between carboxyl groups, hydroxyl groups, amino groups, amide groups, ester groups, imide groups, imine groups, mercapto groups, nitro groups, sulfonate groups, phosphate groups, phosphonate groups, cyano groups, sulfone groups, aldehyde groups, epoxide groups, urethane groups, ketone groups, phenolic groups, aromatic groups, alkyl, alkenyl, alkynyl, acyl and aryl groups, silanol groups, silicon oxide groups, siloxane groups, metal hydroxide groups, metal oxide groups, and elemental metals.

13) Method according to any one of claims 9 to 12, characterised in that the support material is carboxylated polystyrene.

14) Method according to claim 9, characterised in that quantifying in step g) of the fluorescence of the visual marker protein is used in applications selected from analysis, diagnosis (like in enzyme based diagnostic kits), incubation, storage, sensing, arraying and orienting, catalysis, stabilisation, binding, signal transduction, chemical transformation, implant passivation and surface biocompatibilization, surface activation, purification, detoxification and scavenging.

15) A two-component system obtained by any one of claims 9 to 14.

16) A two-component system according to claim 15 described by and an activated support material and a protein encoded by a recombinant plasmid DNA construct according to any one of claims 1 to 8